FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (MODIFIED)	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	**X-11650**
	U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) **10/018099**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/15018	**08 June 2000 (08.06.00)**	**15 July 1999 (15.07.99)**

TITLE OF INVENTION: **PROCESS FOR DEACYLATION OF LIPODEPSIPEPTIDES**

APPLICANT(S) FOR DO/EO/US: **Adam Joseph Kreuzman, Palaniappan Kulanthaivel and Michael John Rodriguez**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [X] A copy of the International Preliminary Examination Report (IPER), including any annexes, and, if not in English, an English language translation of the annexes to the IPER under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/018099	PCT/US00/15018	X-11650

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$740.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee paid to USPTO (37 CRF 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) **$100.00**

CALCULATIONS PTO USE ONLY		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$	**890.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than __20 __30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	14 -20=	0	X $18.00	$	
Independent claims	7 - 3=	4	X $84.00	$	**336.00**
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$	**280.00**
			TOTAL OF ABOVE CALCULATIONS =	$	**616.00**
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
			SUBTOTAL =	$	**1,506.00**
Processing fee of **$130.00** for furnishing English translation later than __ 20 __30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
			TOTAL NATIONAL FEE =	$	**1,506.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property				$	
			TOTAL FEES ENCLOSED =	$	**1,506.00**
			Amount to be refunded	$	
			charged	$	

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed.
b. [X] Please charge my Deposit Account No. 05-0840 in the amount of **$ 1,506.00** to cover the above fees. A duplicate copy of this sheet is enclosed.
c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 05-0840. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
ELI LILLY AND COMPANY
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10 Dec 2001
Date

25885
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PROCESS FOR DEACYLATION OF LIPODEPSIPEPTIDES

FIELD OF THE INVENTION

The present invention relates to lipodepsipeptides, in particular, deacylation of the N-acyl side-chain of pseudomycin and syringomycin natural products and the compounds produced therefrom.

BACKGROUND OF THE INVENTION

Pseudomycins and syringomycins are natural products isolated from liquid cultures of *Pseudomonas syringae* (plant-associated bacterium) and have been shown to have antifungal activities. (see i.e., Harrison, L., et al., "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity," J. Gen. Microbiology, **137**(12), 2857-65 (1991) and US Patent Nos. 5,576,298 and 5,837,685) Unlike the previously described antimycotics from *P. syringae* (e.g., syringomycins, syringotoxins and syringostatins), pseudomycins A-C contain hydroxyaspartic acid, aspartic acid, serine, dehydroaminobutyric acid, lysine and diaminobutyric acid.

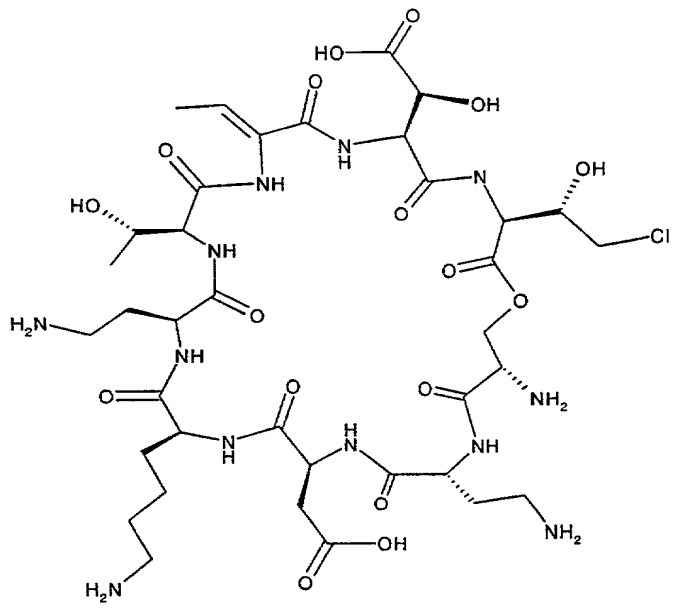
The peptide moiety for pseudomycins A, A', B, B', C, C' corresponds to L-Ser-D-Dab-L-Asp-L-Lys-L-Dab-L-aThr-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl) with the terminal carboxyl group

closing a macrocyclic ring on the OH group of the N-terminal Ser. The analogs are distinguished by the N-acyl side chain, i.e., pseudomycin A is N-acylated by 3,4-dihydroxytetradeconoyl, pseudomycin A' by 3,4-dihydroxypentadecanoyl, pseudomycin B by 3-hydroxytetradecanoyl, pseudomycin B' by 3-hydroxydodecanoyl, pseudomycin C by 3,4-dihydroxyhexadecanoyl and pseudomycin C' by 3-hydroxyhexadecanoyl. (see i.e., Ballio, A., et al., "Novel bioactive lipodepsipeptides from *Pseudomonas syringae*: the pseudomycins," FEBS Letters, **355**(1), 96-100, (1994) and Coiro, V.M., et al., "Solution conformation of the *Pseudomonas syringae* MSU 16H phytotoxic lipodepsipeptide Pseudomycin A determined by computer simulations using distance geometry and molecular dynamics from NMR data," Eur. J. Biochem., **257**(2), 449-456 (1998).)

Pseudomycins and syringomycins are known to have certain adverse biological effects. For example, destruction of the endothelium of the vein, destruction of tissue, inflammation, and local toxicity to host tissues have been observed when pseudomycin is administered intraveneously. Therefore, there is a need to identify compounds within this class that are useful for treating fungal infections without the currently observed adverse side effects.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a process for deacylating the N-acyl side-chain of a lipodepsipeptide natural product to produce the corresponding nucleus. The deacylation of pseudomycin compounds produces the pseudomycin amino nucleus represented by the following structure I.

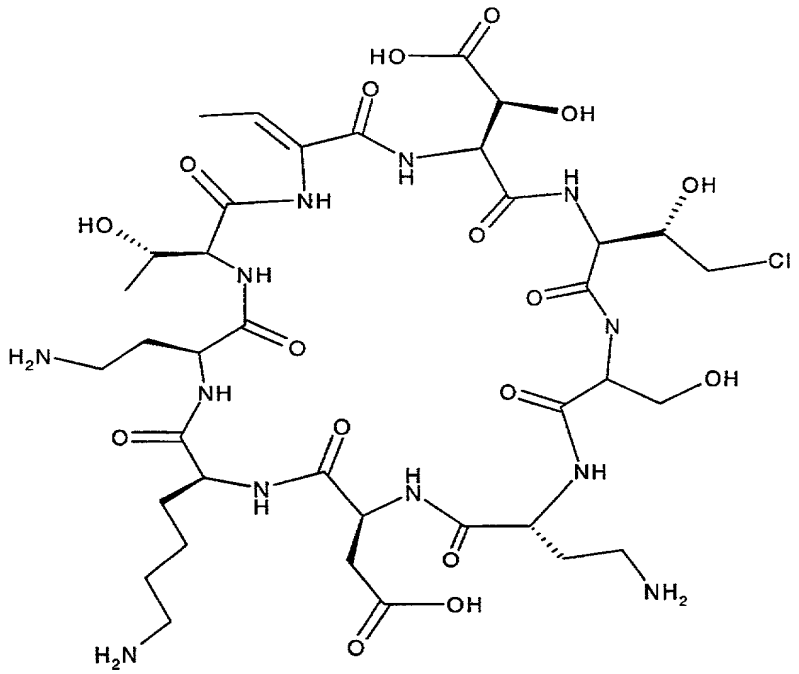


I

The nucleus is useful as a starting material for producing semi-synthetic derivatives of the corresponding natural product.

The process includes reacting a pseudomycin natural product with a deacylase enzyme selected from the group consisting of ECB deacylase and polymyxin acylase to produce

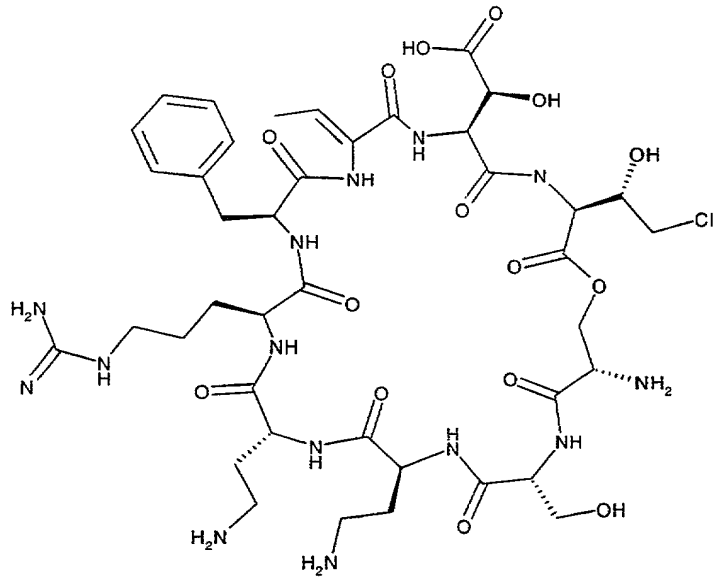
the corresponding nucleus represented by structure I. The free amine may rearrange to produce a cyclic peptide nucleus having a free hydroxy group represented by structure II below (also referred to as pseudomycin hydroxy nucleus).



II

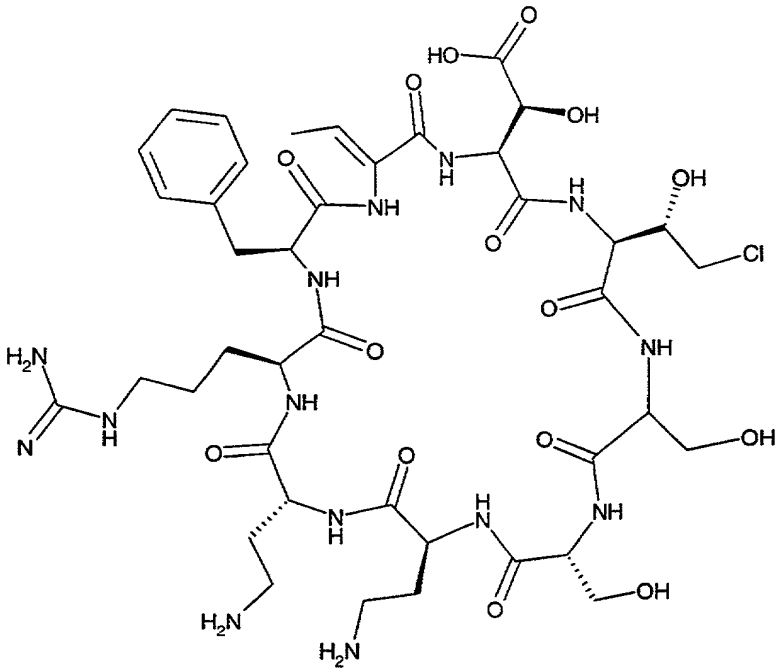
Compound II may then serve as starting material to generate novel derivatives which may be pharmaceutically active.

In another embodiment of the present invention, the process described above is used to deacylate syringomycin compounds to provide a syringomycin amino nucleus. For example, the amino nucleus of Syringomycin E has the following structure III.



III

Like the pseudomycin amino nucleus, the syringomycin amino nucleus may rearrange to form the following Compound IV (also referred to as syringomycin hydroxy nucleus).

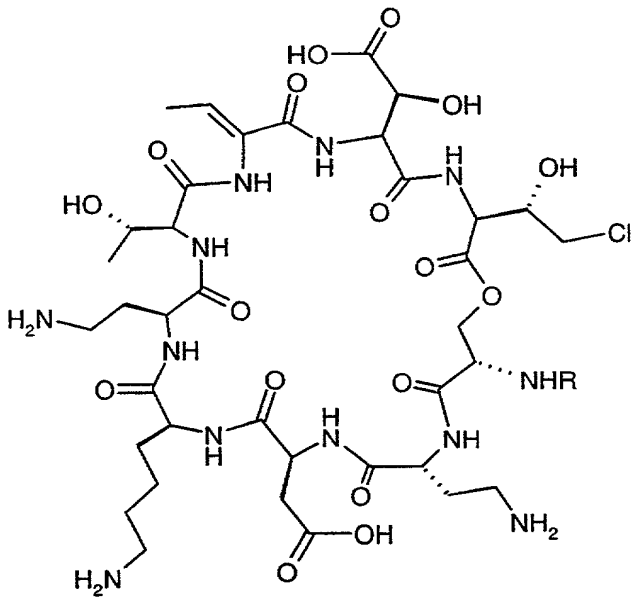


IV

Even though specific chiral forms are depicted above for Compounds I, II, III and IV, other chiral forms are within the spirit of the present invention. Each of the compounds may also exist as pharmaceutically acceptable salts, hydrates or solvates thereof.

Definitions

As used herein, the term "pseudomycin" refers to compounds having the following formula:



where R is a lipophilic moiety. The lipophilic moiety includes C_9-C_{15} alkyl, C_9-C_{15} hydroxyalkyl, C_9-C_{15} dihydroxyalkyl, C_9-C_{15} alkenyl, C_9-C_{15} hydroxyalkenyl, or C_9-C_{15} dihydroxyalkenyl. The pseudomycin compounds A, A', B,

B', C, C' are represented by the formula I above where R is as defined below.

Pseudomycin A R = 3,4-dihydroxytetradecanoyl

Pseudomycin A' R = 3,4-dihydroxypentadecanoyl

Pseudomycin B R = 3-hydroxytetradecanoyl

Pseudomycin B' R = 3-hydroxydodecanoyl

Pseudomycin C R = 3,4-dihydroxyhexadecanoyl

Pseudomycin C' R = 3-hydroxyhexadecanoyl

DETAILED DESCRIPTION OF THE INVENTION

Applicants have discovered a process for enzymatically deacylating the N-acyl side-chain of a broad spectrum of lipodepsipeptide natural products to produce the corresponding nucleus. Surprisingly, the free amine nucleus rearranges to produce the free hydroxy derivative such as the compounds shown above as structures II and IV. Compounds I and III can be converted to Compounds II and IV, respectively, by exposing Compound I or III to a pH $\geq$ 6. If the desired product is Compound I or III, then one could reduce the rate at which the rearranged product forms from the deacylated pseudomycin or deacylated syringomycin with the addition of an acid, such as trifluoroacetic acid. However, the addition of an acid could result in lower yields of the amine nucleus. At lower pHs, the enzyme may precipitate out of the reaction mixture thus stopping the

conversion. Therefore, the pH of the reaction mixture is preferably not lowered less than about 5.5. One could prevent enzyme precipitation by separating the enzyme from the reaction through a molecular weight membrane (i.e., 10,000 to 50,000 molecular weight cutoff). The effluent through the membrane would contain compounds having a molecular weight less than 10,000 to 5,000 (e.g., Compounds I-IV) and would exclude the higher molecular weight enzyme. The effluent could then be pH adjusted down to stabilize the product.

Unlike acid deacylation processes (e.g., trifluoroacetic acid in an aqueous solvent at room temperature), the inventive enzymatic process may be used to deacylate pseudomycin or syringomycin analogs with or without gamma or delta hydroxy side chains. Therefore, the spectrum of starting natural products is expanded significantly. For example, one may deacylate pseudomycin A, A', B, B', C or C' using the inventive process. Whereas, the acid deacylation process is useful only with pseudomycin A, A' and C.

Suitable enzymes include ECB deacylase and Polymyxin acylase (available in both a crude & pure form as 161-16081 Fatty Acylase, Pure and 164-16081 Fatty Acylase, Crude, from Wako Pure Chemical Industries, Ltd.) ECB deacylase can be obtained from *Actinoplanes utahensis* (see e.g., LaVerne, D,

et al, "Deacylation of Echinocandin B by *Actinoplanes utahensis*," *J. of Antibiotics*, 42(3), 382-388 (1989).) The *Actinoplanes utahensis* ECB deacylase enzyme may be purified by the process described in U.S. Patent No. 5,573,936, incorporated herein by reference. One may also use an enzyme that has been cloned and expressed in *Streptomyces lividans*. Attempts to deacylate pseudomycin A with Pen G Amidase and Phthalyl Amidase were not successful.

The enzymatic deacylation may be accomplished using standard deacylation procedures well known to those skilled in the art. For example, general procedures for using Polymyxin acylase may be found in Yasuda, N., et al, *Agric. Biol. Chem.*, 53, 3245 (1989) and Kimura, Y., et al., *Agric. Biol. Chem.*, 53, 497 (1989).

The deacylation process is generally ran at temperatures between about 20°C and about 60°C, preferably between about room temperature (25°C) and about 40°C. Higher temperatures may promote the formation of the rearranged product (Compound II). The enzyme is optimally active at pH 8.0 and at a temperature between about 50°C and 60°C. Although the reaction is faster at the higher pH and higher temperature, more rearranged product may be observed at the higher pH. Therefore, the pH of the reaction is

generally kept between about 5.5 and about 8.0. The reaction time will vary depending upon the pH and the temperature. However, with limiting enzyme concentration and saturated substrate concentration at high temperatures and pH, the reaction is linear through 10 minutes. Since Pseudomcyin A is unstable at higher pHs, deacylation of Pseudomycin A is generally ran at a lower pH (between about 5.0 and 6.0) and temperature (about 25°C). For example, deacylation of Pseudomycin A can be ran in a buffered solution containing 0.05 M KPO_4 and 0.8 M KCl. A saturated level of substrate is generally between about 0.5 mg and about 1 mg per ml of reaction.

As discussed earlier, pseudomycins are natural products isolated from the bacterium *Pseudomonas syringae* that have been characterized as lipodepsinonapetpides containing a cyclic peptide portion closed by a lactone bond and including the unusual amino acids 4-chlorothreonine (ClThr), 3-hydroxyaspartic acid (HOAsp), 2,3-dehydro-2-aminobutyric acid (Dhb), and 2,4-diaminobutyric acid (Dab). Methods for growth of various strains of *P. syringae* to produce the different pseudomycin analogs (A, A', B, B', C, and C') are generally described below and also described in more detail in PCT Patent Application Serial No. PCT/US00/08728 filed by Hilton, et al. on April 14, 2000 entitled "Pseudomycin Production by *Pseudomonas Syringae*," incorporated herein by

reference, PCT Patent Application Serial No. PCT/US00/08727 filed by Kulanthaivel, et al. on April 14, 2000 entitled "Pseudomycin Natural Products," incorporated herein by reference, and U.S. Patent Nos. 5,576,298 and 5,837,685, each of which are incorporated herein by reference.

Isolated strains of *P. syringae* that produce one or more pseudomycins are known in the art. Wild type strain MSU 174 and a mutant of this strain generated by transposon mutagenesis, MSU 16H are described in U.S. Patent Nos. 5,576,298 and 5,837,685; Harrison, et al., "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity," J. Gen. Microbiology, **137**, 2857-2865 (1991); and Lamb et al., "Transposon mutagenesis and tagging of fluorescent pseudomonas: Antimycotic production is necessary for control of Dutch elm disease," Proc. Natl. Acad. Sci. USA, **84**, 6447-6451 (1987).

A strain of *P. syringae* that is suitable for production of one or more pseudomycins can be isolated from environmental sources including plants (e.g., barley plants, citrus plants, and lilac plants) as well as, sources such as soil, water, air, and dust. A preferred stain is isolated from plants. Strains of *P. syringae* that are isolated from environmental sources can be referred to as wild type. As

used herein, "wild type" refers to a dominant genotype which naturally occurs in the normal population of *P. syringae* (e.g., strains or isolates of *P. syringae* that are found in nature and not produced by laboratory manipulation). Like most organisms, the characteristics of the pseudomycin-producing cultures employed (*P. syringae* strains such as MSU 174, MSU 16H, MSU 206, 25-B1, 7H9-1) are subject to variation. Hence, progeny of these strains (e.g., recombinants, mutants and variants) may be obtained by methods known in the art.

Mutant strains of *P. syringae* are also suitable for production of one or more pseudomycins. As used herein, "mutant" refers to a sudden heritable change in the phenotype of a strain, which can be spontaneous or induced by known mutagenic agents, such as radiation (e.g., ultraviolet radiation or x-rays), chemical mutagens (e.g., ethyl methanesulfonate (EMS), diepoxyoctane, N-methyl-N-nitro-N'-nitrosoguanine (NTG), and nitrous acid), site-specific mutagenesis, and transposon mediated mutagenesis. Pseudomycin-producing mutants of *P. syringae* can be produced by treating the bacteria with an amount of a mutagenic agent effective to produce mutants that overproduce one or more pseudomycins, that produce one pseudomycin (e.g., pseudomycin B) in excess over other pseudomycins, or that produce one or more pseudomycins under advantageous growth

conditions. While the type and amount of mutagenic agent to be used can vary, a preferred method is to serially dilute NTG to levels ranging from 1 to 100 µg/ml. Preferred mutants are those that overproduce pseudomycin B and grow in minimal defined media.

Environmental isolates, mutant strains, and other desirable strains of *P. syringae* can be subjected to selection for desirable traits of growth habit, growth medium nutrient source, carbon source, growth conditions, amino acid requirements, and the like. Preferably, a pseudomycin producing strain of *P. syringae* is selected for growth on minimal defined medium such as N21 medium and/or for production of one or more pseudomycins at levels greater than about 10 µg/ml. Preferred strains exhibit the characteristic of producing one or more pseudomycins when grown on a medium including three or fewer amino acids and optionally, either a lipid, a potato product or combination thereof.

Recombinant strains can be developed by transforming the *P. syringae* strains, using procedures known in the art. Through the use of recombinant DNA technology, the *P. syringae* strains can be transformed to express a variety of gene products in addition to the antibiotics these strains produce. For example, one can modify the strains to

introduce multiple copies of the endogenous pseudomycin-biosynthesis genes to achieve greater pseudomycin yield.

To produce one or more pseudomycins from a wild type or mutant strain of P. syringae, the organism is cultured with agitation in an aqueous nutrient medium including an effective amount of three or fewer amino acids, preferably glutamic acid, glycine, histidine, or a combination thereof. Alternatively, glycine is combined with one or more of a potato product and a lipid. Culturing is conducted under conditions effective for growth of *P. syringae* and production of the desired pseudomycin or pseudomycins. Effective conditions include temperatures from about 22ºC to about 27ºC, and a duration of about 36 hours to about 96 hours. Controlling the concentration of oxygen in the medium during culturing of *P. syringae* is advantageous for production of a pseudomycin. Preferably, oxygen levels are maintained at about 5 to 50% saturation, more preferably about 30% saturation. Sparging with air, pure oxygen, or gas mixtures including oxygen can regulate the concentration of oxygen in the medium.

Controlling the pH of the medium during culturing *of P. syringae* is also advantageous. Pseudomycins are labile at basic pH, and significant degradation can occur if the pH of the culture medium is above about 6 for more than about 12 hours. Preferably, the pH of the culture medium is

maintained between 6 and 4. *P. syringae* can produce one or more pseudomycins when grown in batch culture. However, fed-bath or semi-continuous feed of glucose and optionally, an acid or base (e.g., ammonium hydroxide) to control pH, enhances production. Pseudomycin production can be further enhanced by using continuous culture methods in which glucose and ammonium hydroxide are fed automatically.

Choice of *P. syringae* strain can affect the amount and distribution of pseudomycin or pseudomycins produced. For example, strains MSU 16H and 67 H1 each produce predominantly pseudomycin A, but also produce pseudomycin B and C, typically in ratios of 4:2:1. Strain 67 H1 typically produces levels of pseudomycins about three to five fold larger than are produced by strain MSU 16H. Compared to strains MSU 16H and 67 H1, strain 25-B1 produces more pseudomycin B and less pseudomycin C. Strain 7H9-1 are distinctive in producing predominantly pseudomycin B and larger amount of pseudomycin B than other strains. For example, this strain can produce pseudomycin B in at least a ten fold excess over either pseudomycin A or C.

As discussed earlier, the process described herein is also useful for deacylating syringomycin compounds. Syringomycin E, syringotoxin B, and syringostatin A may be produced from cultures of *Pseudomonas syringae* pv. *syringae* strains B301D, PS268, and SY12, respectively. Syringomycin

A_1 and G may be isolated from *Pseudomonas syringae* pv. *syringae* as well. Strains B301D and PS268 are grown in potato dextrose broth as described by Zhang, L., and J. Y. Takemoto, "Effects of Pseudomonas syringae phytotoxin, syringomycin, on plasma membrane functions of Rhodotorula pilimanae," Phytopathol. 77(2):297-303 (1987). Strain SY12 was grown in syringomycin minimal medium supplemented with 100M arbutin (Sigma Chemical Co., A 4256; St. Louis, Mo.) and 0.1% fructose (SRMAF) (19, 23). SR-E, ST-B, and SS-A are purified by high performance liquid chromatography as described previously by Bidwai, A. P., and J. Y. Takemoto, "Bacterial phytotoxin, syringomycin, induces a protein kinase-mediatedphosphorylation of red beet plasma membrane polypeptides," Proc. Natl. Acad. Sci. USA, 84:6755-6759 (1987). Solubilized AmB containing 35% sodium deoxycholate (Sigma Chemical Co., A 9528; St. Louis, Mo.) and ketoconazole (Sigma Chemical Co., K-1003; St. Louis, Mo.) are used as test standards. A detailed description for the production and isolation of three cyclic lipodepsinonapeptides syringomycin E, syringotoxin B, and syringostatin A may be found in U.S. Patent No. 5,830,855, incorporated herein by reference.

The pseudomycin or syringomycin nucleus or corresponding rearranged compounds (Compounds II and IV) may be isolated and used per se or in the form of its

pharmaceutically acceptable salt or solvate. The term "pharmaceutically acceptable salt" refers to non-toxic acid addition salts derived from inorganic and organic acids. Suitable salt derivatives include halides, thiocyanates, sulfates, bisulfates, sulfites, bisulfites, arylsulfonates, alkylsulfates, phosphonates, monohydrogen-phosphates, dihydrogenphosphates, metaphosphates, pyrophosphonates, alkanoates, cycloalkylalkanoates, arylalkonates, adipates, alginates, aspartates, benzoates, fumarates, glucoheptanoates, glycerophosphates, lactates, maleates, nicotinates, oxalates, palmitates, pectinates, picrates, pivalates, succinates, tartarates, citrates, camphorates, camphorsulfonates, digluconates, trifluoroacetates, and the like.

The term "solvate" refers to an aggregate that comprises one or more molecules of the solute (i.e., pseudomycin and syringomycin compound) with one or more molecules of a pharmaceutical solvent, such as water, ethanol, and the like. When the solvent is water, then the aggregate is referred to as a hydrate. Solvates are generally formed by dissolving the nucleus or rearranged compound (Compounds II or IV) in the appropriate solvent with heat and slowing cooling to generate an amorphous or crystalline solvate form.

EXAMPLES

Biological Samples

P. syringae MSU 16H is publicly available from the American Type Culture Collection, Parklawn Drive, Rockville, MD, USA as Accession No. ATCC 67028. *P. syringae* strains 25-B1, 7H9-1, and 67 H1 were deposited with the American Type Culture Collection on March 23, 2000 and were assigned the following Accession Nos.:

25-B1	Accession No. PTA-1622
7H9-1	Accession No. PTA-1623
67 H1	Accession No. PTA-1621

Chemical Abbreviations

The following abbreviations are used through out the examples to represent the respective listed materials:

ACN - acetonitrile
TFA - trifluoroacetic acid
DMF - dimethylformamide

Example 1

Example illustrates the deacylation of Pseudomycin A using ECB Deacylase enzyme.

Pseudomycin A (50 µg) and purified ECB Deacylase (50 µl) in 900 µl of an aqueous buffer solution containing 0.05 M potassium phosphate and 0.8 M potassium chloride. The pH remained between 6.0 and 8.0. The temperature was increased

from 25°C to 40°C. The reaction was monitored by HPLC (Waters C18 μBondapak 3.9 X 300 mm column, 235 nm, 1% acetonitrile/0.2% trifluoroacetic acid (4 minutes) to 60% acetonitrile/0.2% trifluoroacetic acid (16 minutes)). Both the pseudomycin amine nucleus (Compound I) and the rearranged pseudomycin hydroxy nucleus (Compound II) were observed.

Both Compounds **I** and **II** showed identical M+H ion (m/z 981.3) in the electrospray ionization mass spectroscopy (ESIMS) corresponding to a molecular formula of $C_{37}H_{61}ClN_{12}O_{17}$. (See Table I below) Detailed analysis of ^1H and TOCSY (total correlation spectroscopy) NMR spectra enabled the assignment of all protons for the hydrolysis products which supports structures I and II. The ^1H NMR chemical shifts of the β-protons (4.83 and 4.46 ppm) of the serine residue of **I** were consistent with those found in pseudomycins A, B and C, indicating that the peptide macrocycle was intact. Furthermore, as expected, the TOCSY spectrum did not show the typical amide proton as part of the serine spin system. On the other hand, in II the serine β-protons underwent considerable upfield shifts (3.78 and 3.74 ppm) suggesting that these protons were not bearing the lactone functionality. This and the fact that the β-protons, in addition to the α proton, correlated to an amide

proton at 8.04 ppm in the TOCSY spectrum indicated that the lactone of the macrocycle rearranged to a peptide core as depicted in II.

Table I

^{1}H NMR data[a] of **I** and **II** in H_2O+CD_3CN

Amino Acid	Position	I	II
Ser	NH	-	8.04
	α	4.30	4.30
	β1	4.83	3.78
	β2	4.46	3.74
Dab-1[b]	NH	9.19	7.99
	α	4.06	4.19
	β1	2.03	2.15
	β2		2.01
	γ1	3.03	2.92
	γ2	2.96	
Asp	NH	8.51	8.20
	α	4.61	4.56
	β1	2.89	2.84
	β2	2.83	2.75
Lys	NH	7.90	8.11
	α	4.23	4.06
	β1	1.79	1.76
	β2	1.71	1.68
	γ1	1.27	1.30
	γ2		1.25
	δ	1.54	1.54
	ε	2.84	2.84
	NH_2	7.34	7.34
Dab-2[b]	NH	8.35	8.31
	α	4.29	4.34
	β1	2.14	2.09
	β2	1.98	1.91
	γ	2.90	2.92
	NH_2	7.53	7.49
Thr	NH	7.73	7.74
	α	4.24	4.21
	β	3.98	3.98
	γ	1.18	1.16

Table I (continued)

Amino Acid	Position	I	II
Dhb	NH	9.65	9.26
	β	6.69	6.62
	γ	1.69	1.66
OHAsp	NH	7.82	7.83
	α	4.95	4.99
	β	4.72	4.75
ClThr	NH	7.92	7.95
	α	4.90	4.62
	β	4.27	4.25
	γ1	3.48	3.57
	γ2	3.42	3.51

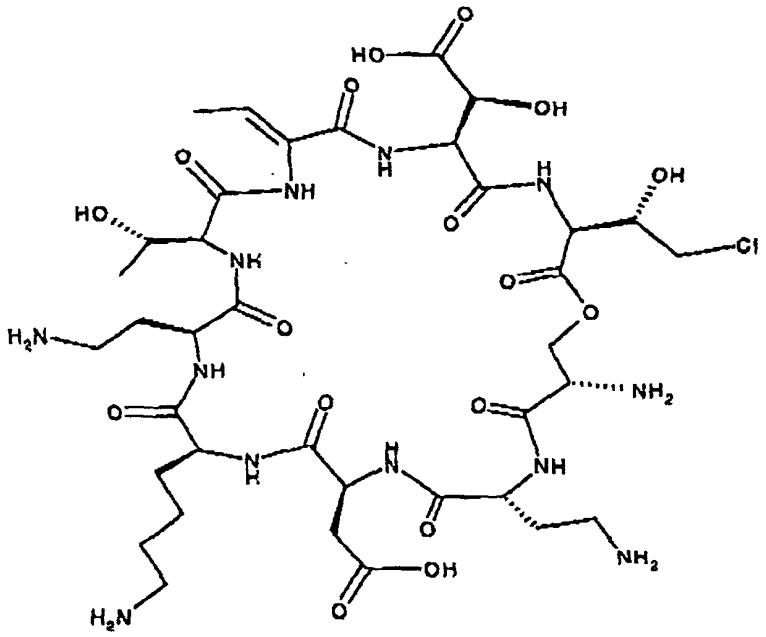
[a] Chemical shifts reported are relative to solvent signal (1.94 ppm).
[b] Assignments may be interchanged.

Other pseudomycin or syringomycin compounds having an N-acyl group may be deacylated using the same general procedures described above.

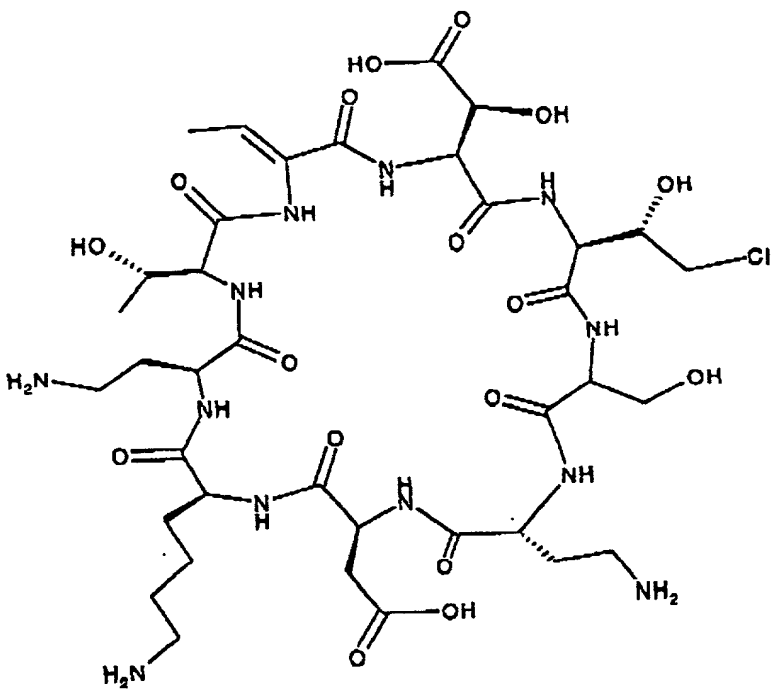
WE CLAIM:

1. A process for deacylating an N-acyl side-chain of a pseudomycin comprising the step of reacting a pseudomycin with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase to produce a pseudomycin nucleus.

2. The process of Claim 1 wherein said pseudomycin nucleus is represented by either structure I or II



I

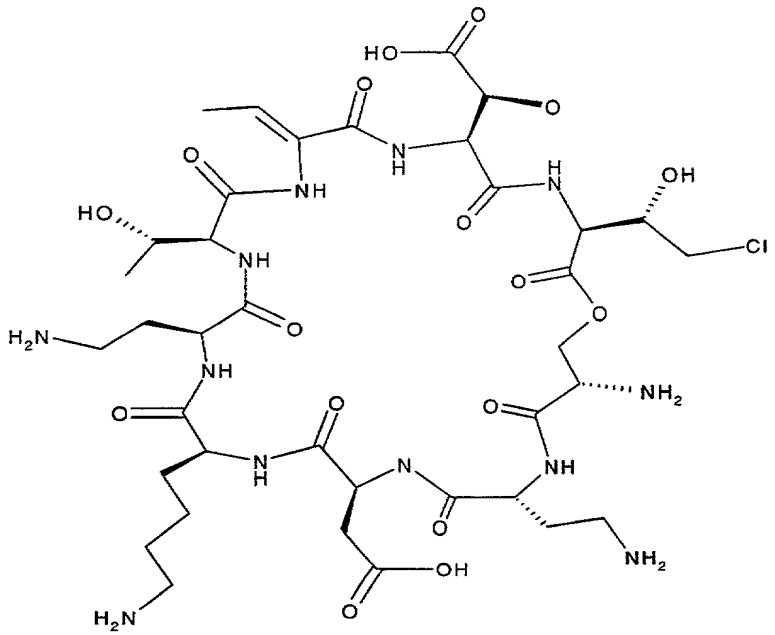


II

or a pharmaceutically acceptable salt, hydrate or solvate thereof.

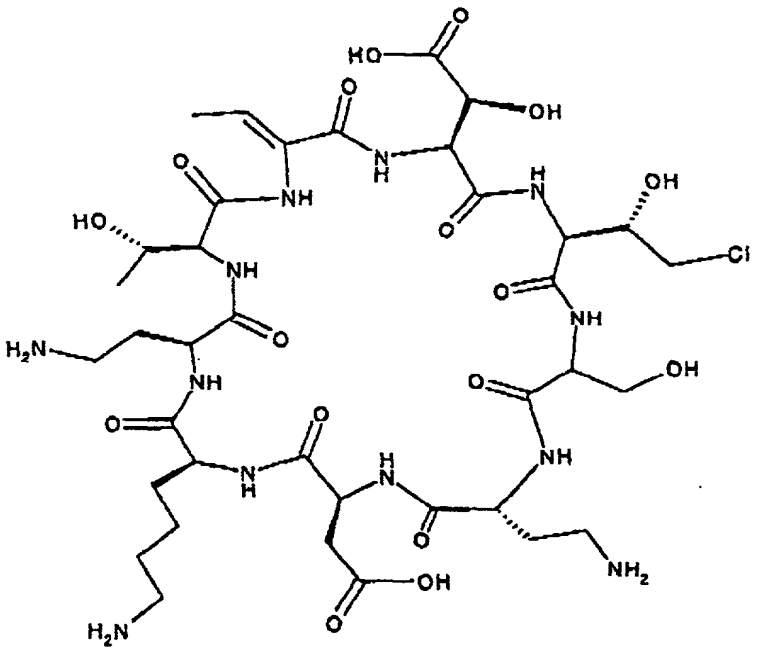
3. The process of Claim 1 wherein said pseudomycin is selected from the group consisting of pseudomycin A, A', B, B', C, and C'.

4. A compound having the following structure



or a pharmaceutically acceptable salt, hydrate or solvate thereof, prepared by the process of Claims 1, 2 or 3.

5. A compound having the following structure



or a pharmaceutically acceptable salt, hydrate or solvate thereof.

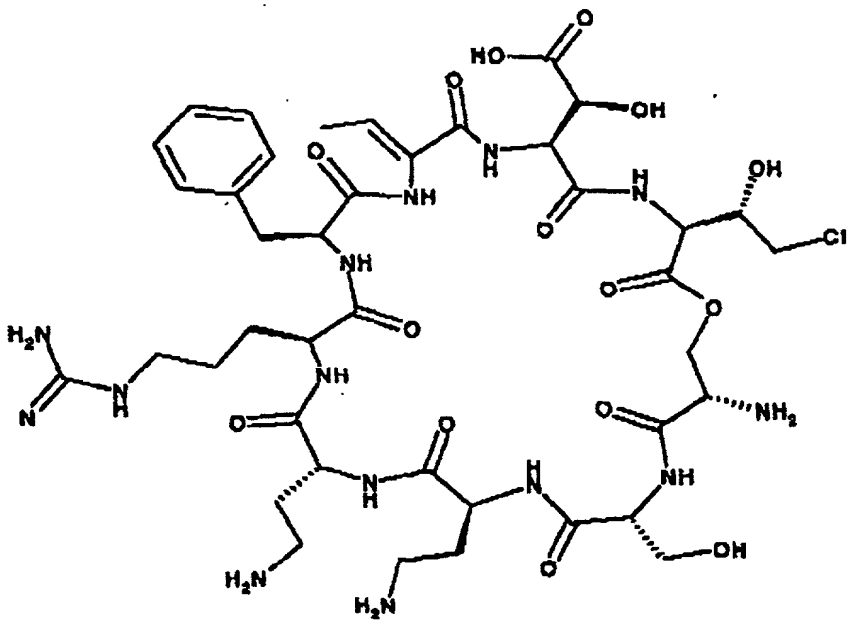
6. A pseudomycin nucleus prepared by reacting a pseudomycin with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase.

7. The pseudomycin nucleus of Claim 6 wherein said pseudomycin is selected from the group consisting of pseudomycin A, A', B, B', C, and C'.

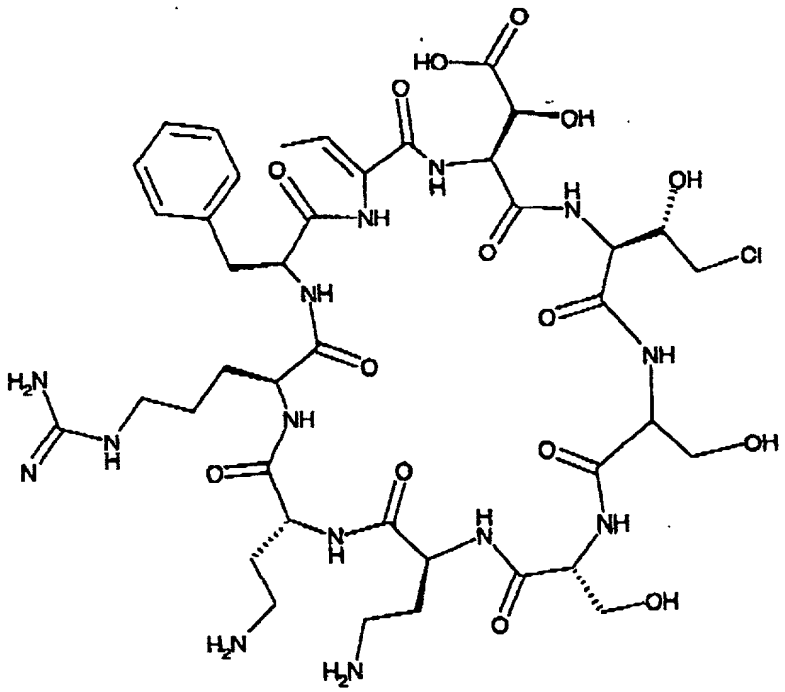
8. A process for deacylating an N-acyl side-chain of a syringomycin comprising the step of

reacting a syringomycin ~~natural product~~ with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase to produce a syringomycin nucleus.

9. The process of Claim 7 wherein said syringomycin nucleus is represented by either structure III or IV



III

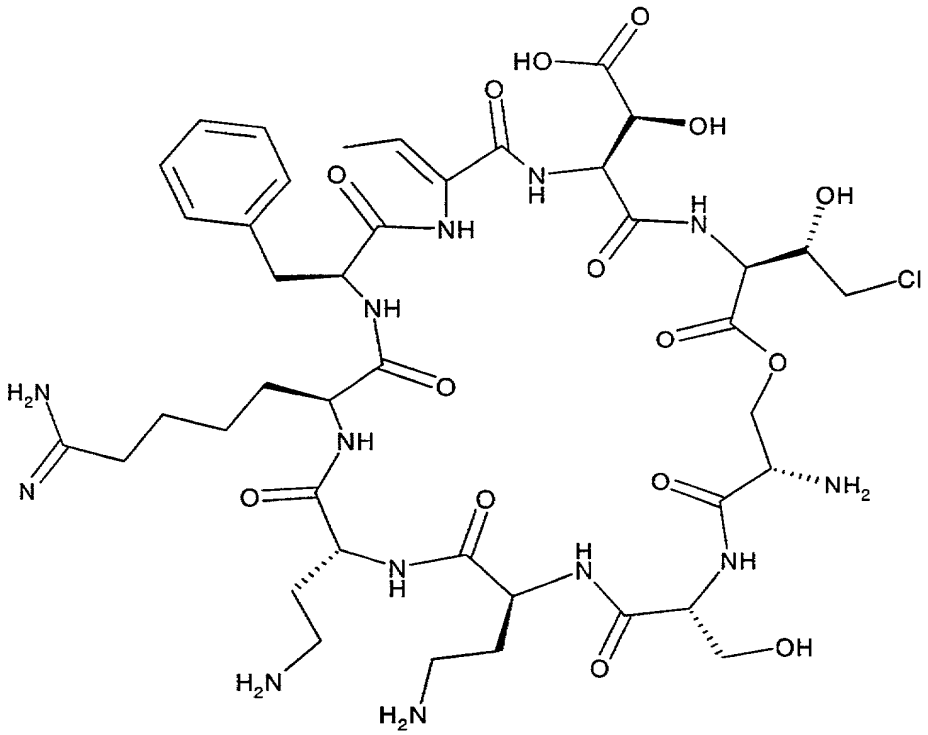


IV

or a pharmaceutically acceptable salt, hydrate or solvate thereof.

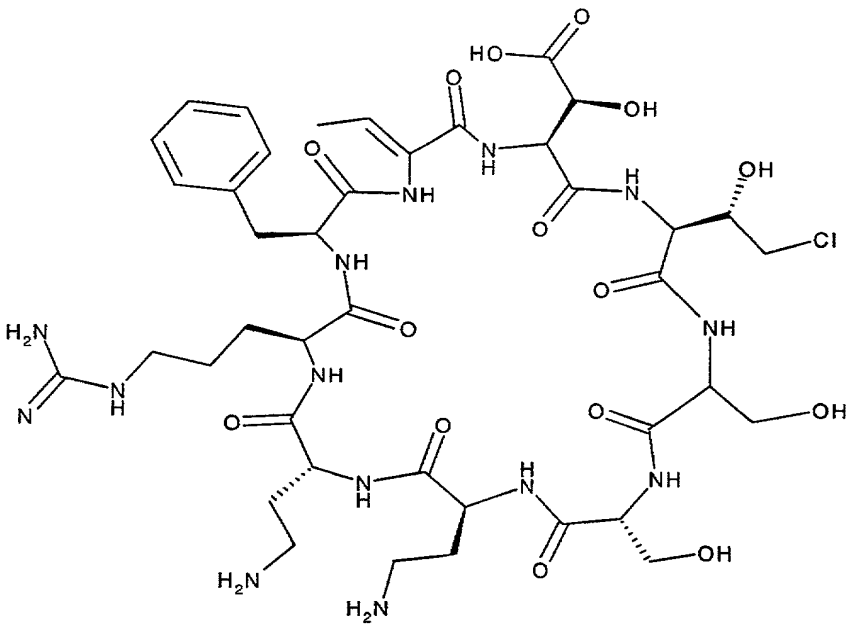
10. A syringomycin nucleus prepared by reacting a syringomycin with a deacylating enzyme selected from the group consisting of ECB deacylase and polymyxin acylase.

11. A compound having the following structure



or a pharmaceutically acceptable salt, hydrate or solvate thereof.

12. A compound having the following structure



or a pharmaceutically acceptable salt, hydrate or solvate thereof.

Please type a plus sign (+) inside this box [+]

PTO/SB/01 (8-96) (MODIFIED)

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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing

[ ] Declaration Submitted after Initial Filing

Attorney Docket Number	**X-11650**
First Named Inventor	**Adam Joseph Kreuzman**
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

As a below named Inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole Inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the Invention entitled:

PROCESS FOR DEACYLATION OF LIPODEPSIPEPTIDES

the specification of which
[ ] is attached hereto
OR
[X] was filed on (MM/DD/YYYY) 08 June 2000 as United States Application Number or PCT International Application Number PCT/US00/15018 and was amended on (MM/DD/YYYY) 12 July 2001 (if applicable).

II hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or Inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached YES	Certified Copy Attached NO
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional applications(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/143,968	15 July 1999

[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

Please type a plus sign (+) inside this box [+]
☐

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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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Lawrence T. Welch	29,487
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Direct all correspondence to:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ **A Petition has been filed for this unsigned inventor**

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[X] **Additional Inventors are being named on supplement sheet(s) attached hereto.**

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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION

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Name of Additional Joint Inventor, if any:		[ ] A Petition has been filed for this unsigned inventor					
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Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address							
Post Office Address	SAME AS ABOVE						
City		State		Zip		Country	